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# Growth response of *Salmonella typhimurium* as a function of temperature, pH, organic and inorganic acids, and NaCl concentration

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## Abstract

*Salmonella* is still one of the most important pathogens related with foodborne outbreaks.This study was designed to examine the combined effects of different levels of pH (7.4, 6.4, and 5.4), acids (acetic, citric and hydrochloric acid), temperatures (35 ˚C and 25 ˚C), and NaCl concentrations (0.5, 3 and 6%, w/v), on growth of *Salmonella typhimurium* in brain heart infusion broth. The experiment conducted in triplicate. Growth was monitored by visible turbidity over a 30-day period.To evaluate the effects of explanatory variable on time to detect (TTD) the bacterial growth, parametric survival models based on the weibull distribution was used**.** According to our results, the growth of *S.*typhimurium was affected significantly (*P*<0.05) by selected parameters.TTD for combinations with acetic and citric acid was 6.97 and 1.30 times greater than HCl respectively. TTD for combinations with pH levels of 6.4 and 5.4 was 1.72 and 7.96 times greater than those with pH level of 7.4. This time with 3% and 6% NaCl concentration was 1.38 and 1.82 times greater than with 0.5% NaCl. Furthermore, this period for combinations with incubation temperature of 25°C was 1.30 times greater than combinations with those by incubation temperature of 35°C.The final model showed that all explanatory variables had significant association with time of detection.

**Keywords:** *Salmonella typhimurium,* modeling, predictive microbiology, time to detection

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## Introduction

Salmonellas are now established as one of the most important causes of foodborne illness worldwide. *Salmonella* species have been recognized for over 100 years now as the cause of illnesses ranging from mild to severe food poisoning (gastroenteritis), and even more severe typhoid (enteric fever), paratyphoid, bacteraemia and septicemia (Blackburn and McClure, 2002).

One of the most common causes of food poisoning by *Salmonella* species is due to *S.* typhimurium. In humans *S.* typhimurium does not cause severe disease as *S.* yyphi, and is not normally fatal. The disease is characterized by diarrhea, abdominal cramps, vomiting and nausea, and generally lasts up to 7 days. Unfortunately, in immune-compromized people, that is the elderly, young, or people with depressed immune systems, *Salmonella* infections are often fatal if they are not treated with antibiotics (Adams and Moss, 2008).

Several years ago, hurdle technology was developed as a new concept for the realization of safe, stable, nutritious, tasty, and economical foods (McMeekin *et al*., 2000). Several environmental factors such as temperature, pH, water activity ($a_w$), atmosphere, and presence or absence of preservatives affect the growth of microorganisms in foods. It has been known that combinations of inhibitory factors can give synergistic effects. It is thought that a multi-factorial approach for food preservation may be more successful than a more extreme use of any single treatment (Thomas *et al.,* 1991).

Mathematical model is an equation that describes or predicts the growth, survival or death of microorganisms in foods. The model simply relates the microbial growth, survival or death responses to the levels of the controlling factors throughout the experimental design space. They tell us nothing about the physiological mechanisms or biological, chemical or physical principles that drive the responses (Blackburn and McClure, 2002).

In recent years, modeling the behavior of microorganisms at the growth/no growth interface has been recognized as an important component of modern predictive microbiology (McMeekin *et al*., 1997, McMeekin *et al*., 2000, McMeekin *et al*., 2002).

Microbial growth models are typically developed when the objective is to understand the responses of microorganisms when part of the range of conditions studied permits growth to occur. Suchmodels can describe the increase in numbers with time (kineticmodels), the conditions allowing growth or no growth (boundarymodels), or the chance of growth (probabilistic models) **(**Stewart *et al*., 2002**)**.

Nowadays, there is a greater tendency at the side of consumers in use of additive-free, fresher and more natural tasting food products, while maintaining microbiological safety. The use of natural antimicrobial compounds belonging to the preservatives is more interested in the food industry. Organic acids and aromatic compounds belong to this type of additives, as well as some salts (Kobilinsky *et al*., 2007).

The aim of this study was to model the effects of controlling factors of temperature, pH, NaCl concentration, and type of acids on time of detection of *S.*typhimurium.

## Materials and Methods

*Experimental design*

To assess the effects of different type of acids, pH, sodium chloride (NaCl) concentrations and temperature on growth initiation of *S.*typhimurium, the experiment was arranged in a factorial design in Brain Heart Infusion (BHI) broth. This design (3 × 3 × 3 × 2) included three type of acids (acetic and citric acid as organic acids and hydrochloric acid as aninorganic one), three levels of pH (7.4, 6.4, and 5.4), three levels of NaCl concentrations (0.5, 3 and 6%, w/v), and twostorage temperatures (35°C, and 25°C), and also repeated observations (90 times) for growth in BHI broth during 30 days. The experiment was conducted in triplicate.

*Test organism*

*Salmonella* typhimurium ATCC 14028 (Mast International Inc-England) was used as

the test organism in this study.

*Preparation of inoculums*

The reference bacteria were plated in BHI agar and incubated at 37°C for 24h. Inoculums were prepared by transferring a loop full of the bacterial colonies to isotonic saline solution in a sterile tube to adjust the visual turbidity equal to 0.5 McFarland. This adjustment gave a cell concentration of $1.5 \times 10^8$cfu $ml^{-1}$.

*Performing the Experiment*

BHI powder (3.7g) was dissolved in 100ml of distilled water in a 250ml flask by mild heating. NaCl was added and dissolved to satisfy the experimental design.Then pH was adjusted using normal solution ofacetic acid, citric acid, and hydrochloric acid to pH values of 7.4, 6.4 and 5.4 in designated experiments. The pH measurement was done by pH meter (Jenway, Ltd England). The content of each flask was autoclaved at 121°C for 15 min. The content of flask containing sterile BHI broth was dispensed in portions of 3 ml in to sterile caped tubes (Becton Dickinson 16× 100 mm).

The tubes were inoculated with *S.*Typhimurium suspension to obtain$10^4$ cfu $ml^{-1}$. For each combination the tubes were incubatedat 35 and 25 ˚C for up to 30 days. During these periods all the tubes were observed for visible growth (turbidity) at 8 hour intervals. The date and the number of tubes (combinations) showing growth at a particular observationwere recorded. For each combination a negative control (uninoculated tube) was used. All experiments were conducted in independent triplicate.

*Statistical Analysis*

The outcome variable for consideration in present study was the time of visible growth. Since some combinations did not grow until the end of the study (30 days), standard regression methods were realized inappropriate. Instead, event-time (survival) analysis were employed which were able to use all the experimental data irrespective of whether or not growth occurred.

Parametric survival model based on accelerated failure time (AFT) approach (Kleinbaum and Klein, 2005) was used to quantify the effect of each of the prescribed explanatory variables on time of detection of bacterial growth. The general form of the accelerated failure time model is:

$$\log(t)=\left(\alpha+\beta_1 x_{1i}+...+\beta_m x_{mi}\right)+\log(\tau) \quad \text{Equation 1}$$

Where log ($t$) is the natural logarithm of the time to ‘failure’ (growth), $\alpha$ an intercept term,$\beta_1 x_{1i} + \ldots + \beta_m x_{mi}$ is a linear combination of the *m* explanatory variables and their regression coefficients, and log(τ) is an error term. Using this approach the accelerated failure time coefficients represent the expected change in log (t) for changes in the predictor levels.

In present study, we evaluated the exponential, Weibull, log-normal and log-logistic distributions that can be interpreted in the AFT metric. To evaluate fitness of candidate distribution to the current data the mean square error (MSE) was compared. The smaller MSE value indicates a better fit.

$$MSE = \frac{\sum(\text{predicted} - \text{observed})^2}{n - p} \quad \text{Equation 2}$$

N is the number of observations and p is the number of parameters to be estimated.

To select those explanatory variables that best explained time of detection a backward stepwise approach was used. Explanatory variables that were not statistically significant were removed from the model one at a time, beginning with the least significant, until the estimated regression coefficients for all retained variables were significant at an alpha level of <0.05.All analyses were carried out using Stata Statistical Software, version 10.

## Results

*Evaluation of Growth/No growth*

About 88.88% of combinations (144 out of 162) grew during the study period and 11.12% of combinations (18 out of 162) did not grow and considered as censored observations. All of the combinations that did not grow till the end of study were those with pH level of 5.4 adjusted with acetic acid.

*Evaluation of Time of Detection of Bacterial Growth*

On the basis of MSE value the weibull model best fit the data. The MSE value of the weibull model was 11.78, while the MSE values were 23.9, 23.8 and 13.3 for log-normal, log-logistic and exponential models respectively. Median time of detection of bacterial growth was 1.33 days. Kaplan-Meier survival curve for different types of acidis is presented in Figure 1.

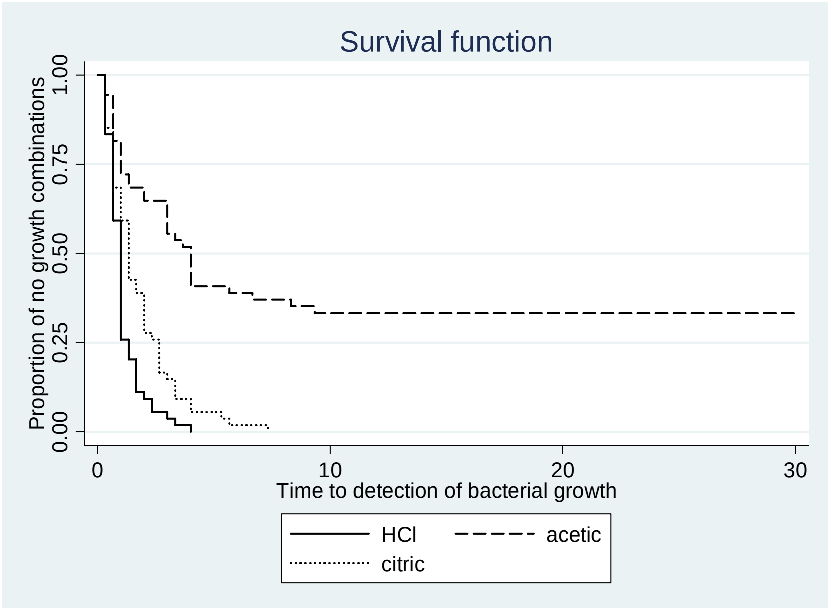



**Figure 1.Kaplan-meier survival curves showing the proportion of no growth combinations for different types of acids.**

**Table1. Accelerated failure time model of factors influencing time of detection of bacterial growth.**

| Variables | $\beta$ (SE) | P-value | Time ratio (95% CI) |
|---|---|---|---|
| Intercept | -1.15(0.126) | < 0.01 | |
| Type of acid: | | | |
| HCl | 0 | | 1 |
| Acetic | 1.94(0.148) | < 0.01 | 6.97 (5.20-9.32) |
| Citric | 0.27(0.109) | 0.015 | 1.30 (1.05-1.62) |
| pH: | | | |
| pH 7.4 | 0 | | 1 |
| pH 6.4 | 0.54(0.112) | < 0.01 | 1.72 (1.38-2.15) |
| pH 5.4 | 2.07(0.149) | < 0.01 | 7.96 (5.94-10.68) |
| Nacl concentration: | | | |
| 0.5 | 0 | | 1 |
| 3 | 0.32(0.116) | 0.005 | 1.38 (1.10-1.73) |
| 6 | 0.60(0.115) | < 0.01 | 1.82 (1.45-2.28) |
| Temperature: | | | |
| Temperature 35 | 0 | | 1 |
| Temperature 25 | 0.26(0.935) | 0.005 | 1.30 (1.08-1.56) |
| p | 1.81 (0.121) | | |
| 1/p | 0.55 (0.037) | | |

The final model showed that all explanatory variables had significant association with time of detection (Table 1). On average, time of detection for combinations with acetic acid and citric acid was 6.97 and 1.30 times greater than HCl respectively.TTD for those combinations with pH levels of 6.4 and 5.4 was 1.72 and 7.96 times greater than those with pH level of 7.4. Also, this time for combinations with 3% and 6% NaCl concentration was 1.38 and 1.82 times greater than combinations with 0.5%NaCl. Furthermore, this period for combinations with incubation temperature of 25°C was 1.30 times greater than combinations with those with incubation temperature of 35°C. Figure 2 shows the observed time of detection of bacterial growth and value that is predicted by Weibull model for time of detection of *S.*typhimurium in designated combinations. The final model equation is shown below:

$$TTD = [-\ln 0.5]^{\frac{1}{1.81}} \times e^{-1.15+0.26T_{25}+0.32NaCl_3+0.6NaCl_6+0.54PH_{6.4}+1.94\text{Acetic acid}+0.27\text{Citric acid}}$$

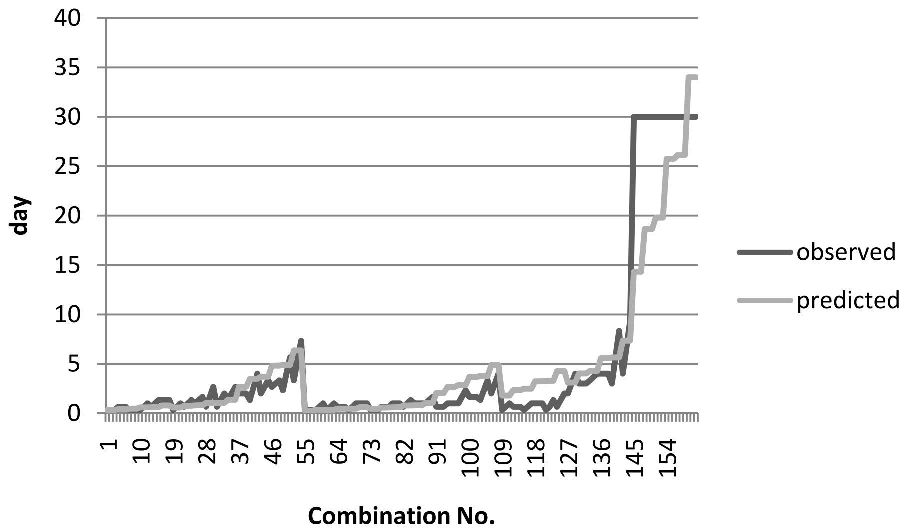



**Figure 2. Observed and predicted time needed for growth initiation of *S.typhimurium* (TTD) according to the weibull model.**

## Discussion

Predictive microbiology combines mathematical modeling with experimental data on combinations of factors that influence the growth of food spoilage and/or food-borne pathogenic microorganisms (Tienungoon *et al*., 2000). Some common hurdles used to control microbial growth are pH, sodium chloride, and storage temperature.

A clear understanding of risk factors is very important to develop appropriate prevention and control strategies for infection causedby such pathogens as *S.*typhimurium. The ability of *S.* typhimurium to grow in foods and laboratory media under a variety of conditions has been quantified and reviewed (Akhoondzadeh Basti and Razavilar, 2004, Moosavy *et al*., 2008, Buzrul and Alpas 2007, Dubal *et al*., 2004, Erkmen, 2009**,** Greenacre and Brocklehurst, 2006, Nazer *et al*., 2005, Wan Norhana *et al*., 2010, Pin *et al*., 2010, Theys *et al*., 2008, Gibson *et al*., 1988).

Akhoondzadeh Basti and Razavilar (2004) showed that the TTD of *S.* typhimurium was affected significantly ($p$<0.05) by the values of potassium sorbate, pH and temperature; but the TTD value did not affected by NaCl concentration in their study.

In the present study, we used the concepts of the TTD in a factorial design study to

quantify the effect of types of acid, pH, temperature and NaCl concentration on the growth responses of *S.* typhimuriumin BHI broth. It has been shown that in the final model (in this study) all explanatory variables had significant association with time of detection ($p<0.05$).

In our study, the values of the TTD were higher at low levels of temperatures, pH (especially when adjusted by acetic acid) and high levels of NaCl concentrations.

Mathematical models describing the growth of microorganisms as a function of temperature, pH and organic acid concentration can be a helpful tool to determine the environmental conditions that will ensure food safety (Le Marc *et al*., 2002).

One of the important food preservation techniques is reducing the pH by adding acids. Weak acids are popular food preservatives because they are effective at low concentrations and can lower the pH sufficiently to reduce the growth of pathogenic bacteria (Presser *et al*., 1998).

Partial dissociation of weak acids, such as acetic acid, plays an important role in their ability to inhibit microbial growth. It is well established that, although addition of strong acids has a more profound effect on pH, they are less inhibitory than weak lipophilic acids at the same pH. This is because microbial inhibition by weak acids is not solely due to the creation of a high extracellular proton concentration, but is also directly related to the concentration of undissociated acid (Martin and Moss, 2008).

The pH minima of certain bacteria have been shown to be dependent on the type of acid used. Citric, hydrochloric, phosphoric, and tartaric acids permit growth at a lower pH value than acetic or lactic acids (Jay *et al*., 2005).

Corner *et al*., (1997) and Cutter*et al*., (1996) have shown that acetic acid can be effective against *Escherichia coli* O157:H7 and *S.*typhimurium on bovine carcass.

In our study the values of TTD for combinations with acetic acid and citric acid was 6.97 and 1.30 times greater than HCl respectively. The bactericidal effect of acetic acid has been demonstrated by its action on certain pathogens. When two species of *Salmonella* were added to an oil-and-vinegar-based salad dressing, the initial inoculums of 5 × $10^6$*S.*enteritidis could not be detected after 5 minutes nor could *S. typhimurium* be detected after 10 minutes (Miller and Martin, 1990).

It is not surprising that the growth and metabolism of micro-organisms are influenced by pH because acidity or alkalinity of an environment has a profound effect on the activity and stability of macromolecules (Adams and Moss, 2008).

In this study our results revealed that the time of detection (TTD) for those combinations with pH levels of 6.4 and 5.4 was 1.72 and 7.96 times greater than those with pH level of 7.4. Therefore according to our results, decreasing the pH of broth medium had a significant effect on growth initiation of *S*. typhimurium.

Reducing water activity is another widely used food preservation technique, and the combination of reduced water activity, reduced pH, and weak organic acids is a key element in the stability of many shelf-stable foods (Presser *et al*., 1998). The widespread addition of NaCl to food also creates a need to elucidate the responses of potential pathogens to elevate NaCl concentrations.

In our model we used NaCl as humectant to depress $a_w$. Microbial survival and/or growth depend not only on $a_w$but also on the chemical and physical properties of the humectant (Stewart *et al.,* 2002). Similar to the pH effect on microbial growth behavior, which depends on the acidulant, several authors have reported that the growth boundaries for various genera of microorganisms differ depending on thetype of humectant used to depress $a_w$ rather than the absolutevalue of $a_w$ (Chirife 1994., Marshall *et al* 1971).

This study indicated that the TTD for combinations with 3% and 6% NaCl concentration was 1.38 and 1.82 times greater than combinations with 0.5% NaCl (Table 1).

Temperature is the most common hurdle used to control microbial growth. According to the results, the growth of *S*. typhimurium was significantly ($p < 0.05$) affected by temperature. As the result showed in Table 1 theinitial visual growth period for combinations with incubation temperature of 25°C was 1.30 times greater than combinations with the same parameters, but incubation temperature of 35°C. According to our results, decreasing the incubation temperature had also a significant effect on growth initiation of inoculated bacteria. Probably the most important effect of temperature on growth of a microorganism is on the shape of enzymes required for metabolism and they will have the proper shape only within a relatively narrow range of temperatures.

Zhao *et al*., (2002) developed linear model with polynomial term for the effects of inoculum size, pH, NaCl and temperature on the TTD of *C. botulinum* 56A. They showed when the NaCl concentration and temperature increased, the TTD was increased and when the pH increased, the TTD was decreased.Present study showed the same results for the effects of the designated factors on the TTD of *S.*Typhimurium.

Figure 2 shows the observed time to detection of bacterial growth and value that was predicted by Weibull model for TTD of *S*. typhimurium in designated combinations. Our designated models adequately predicted the growth initiation, and inhibition conditions of *S*. typhimurium as affected by different levels of pH, temperature, types of acids, and NaCl concentrations. Therefore, obtained predictions showed a good correlation to observations.

Since the experimental data are usually derived from studies using laboratory media, there can be no guarantee that predicted values will match those that would occur in any specific food system. Before the models could be used in such a manner, the user would have to validate the models for each specific food of interest.

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IJVST

# تعیین محدوده رشد سالمونلا تیفی موریوم تحت تاثیر دما، pH، اسید های آلی و غیر آلی و غلظت نمک

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## چکیده

باکتری سالمونلا از مهمترین پاتوژن‌های دخیل در ایجاد موارد شیوع عفونت های حاصل از مواد غذایی محسوب می‌شود. این مطالعه جهت بررسی اثر عوامل موثر بر رشد سالمونلا تیفی موریوم شامل سطوح مختلف pH( ۷/۴، ۶/۴، ۵/۴)، اسیدهای مختلف ( اسید استیک، سیتریک، کلریدریک)، دما (۲۵°C، ۳۵°C)، و غلظت‌های مختلف نمک طعام (۰/۵٪، ۳٪، ۶٪)، در محیط براث قلب و مغز طراحی گردید. مطالعه در سه تکرار انجام شد. رشد میکروارگانیسم بر اساس بررسی کدورت قابل مشاهده طی ۳۰ روز انجام شد. جهت ارزیابی تاثیر متغیرها بر روی زمان ایجاد کدورت قابل مشاهده مدل بقاء بر اساس توزیع وایبال مورد استفاده قرار گرفت. آنالیز آماری نتایج مربوط به داده‌های به‌دست آمده در این مطالعه نشانگر تاثیر معنی‌دار پارامتر های فوق بر روی رشد سالمونلا تیفی موریوم بود. زمان لازم برای ایجاد کدورت در حالت‌های محیط کشت حاوی اسید استیک و اسید سیتریک بترتیب ۶/۹۷ و ۱/۳۰ برابر زمان لازم برای اسید کلریدریک بود. این زمان برای حالت‌های دارای pH ۶/۴ و ۵/۴ به‌ترتیب ۱/۷۲ و ۷/۹۶ برابر زمان لازم برای pH ۷/۴ بود. همچنین زمان لازم برای ایجاد کدورت قابل مشاهده در حالت‌های حاوی ۳ و ۶٪ نمک به‌ترتیب ۱/۳۸ و ۱/۸۲ برابر زمان مربوط به ۰/۵ نمک بود. علاوه بر این زمان مورد نیاز برای ایجاد کدورت در حالت‌های گرمخانه گذاری شده در دمای ۲۵ درجه سانتی‌گراد ۱/۳۰ برابر این زمان برای ۳۵ درجه سانتی‌گراد تعیین شد. مدل نهایی به‌دست آمده نشانگر ارتباط معنی‌دار کلیه متغیرها بر زمان ایجاد کدورت بود.

**واژگان کلیدی:** سالمونلا تیفی موریوم، مدل سازی، میکروبیولوژی پیشگو، زمان ایجاد کدورت